High prevalence of large trematode eggs in schoolchildren in Cambodia

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Abstract

Large trematode eggs (LTE) resembling *Fasciola* spp. eggs were reportedly found in the stools of schoolchildren in Kandal province, Cambodia. This study aimed to assess the prevalence of LTE in the stools of children attending the affected school, identify potential risk factors for infection and ascertain the trematode species. We performed a cross-sectional study involving an in-depth questionnaire administered to schoolchildren at the affected school, and examined cattle droppings in the surrounding area and the livers of slaughtered cattle. Three stool samples were examined per child, using Kato-Katz and formalin-ether concentration techniques. In addition, blood serum ELISA and coprological PCR was conducted for species clarification. Cattle droppings were examined by cup sedimentation and coprological ELISA. LTE were observed in the stools of 228 schoolchildren (46.5%). Two blood serum samples from schoolchildren were positive for *Fasciola hepatica* in a first ELISA but were negative in a confirmation immunofluorescence antibody test. Out of 221 PCR samples, only one tested positive for *Fasciola* spp. and none for *Fasciolopsis buski*. The consumption of raw aquatic plants (OR=3.3) and fermented fish sauce (OR=2.1) were significantly associated with LTE in the stool. *Fasciola* spp. flukes were observed in 18.3% of 191 cattle livers. The prevalence of fascioliasis in cattle droppings was 88.8%. The low prevalence of schoolchildren that tested positive for *Fasciola* spp. with specific molecular diagnostics and that had no diagnostic evidence of *Fasciolopsis buski* strongly indicates that the majority of microscopically observed LTE are from *Echinostoma* spp.. *Fasciola* sp. transmission from cattle to human is possible and public health services need to be alerted accordingly.

2

1. Introduction

In low and middle income countries, intestinal multiparasitism is the rule rather than the exception (McKenzie, 2005; Petney and Andrews, 1998; Steinmann et al., 2010). In Southeast Asia, intestinal helminth and protozoa multiparasitism is well known and has been documented (Lee et al., 2002; Park et al., 2004; Sayasone et al., 2011, 2009; Sinuon et al., 2003). Several endemic species of food-borne trematodes (FBT) have been identified, such as liver flukes (*Opisthorchis viverrini*, and *Fasciola* spp.) and intestinal flukes (*Fasciolopsis buski*, and *Echinostoma* spp.) (Hien et al., 2001; Keiser and Utzinger, 2005; Quang et al., 2008; Sayasone et al., 2011; Sohn et al., 2011a, 2011b). Helminthic multiparasitism poses a serious challenge for parasitological diagnosis, as eggs from different species may morphologically resemble one another. For example, the size, oval shape and presence of small and inconspicuous operculum of *Fasciola* spp. eggs are indistinguishable from *Fasciolopsis buski* and some *Echinostoma* spp. eggs. The average egg size of all three parasite genera is similar in terms of length and width: *Fasciola hepatica* 106.5-171.5/63.9-95.4 µm, *F. gigantica* 150.9-182.2/85.1-106.2 µm (Valero et al., 2009), *Echinostoma revolutum* 97-117/ 61-65 µm (Sohn et al., 2011a); *Echinostoma ilocanum* 89-99/52-58 µm (Sohn et al., 2011b) and *Fasciolopsis buski* µm. They can be characterised as *Fasciola*-like eggs or large trematode eggs (LTE). In 2009, LTE were observed in the stools of 27/150 schoolchildren (18.0%) in the Damrei Chhlang village primary school, Kandal province, Cambodia, during a standard parasitological survey using the Kato-Katz technique (Khieu et al., 2013). Given the high prevalence of *Fasciola* spp. infection among cattle in Southern Cambodia (Tum et al.,

2007), LTE could have been attributable to *Fasciola* spp. infection. However, other trematode species could also have been responsible.

The aim of this study was to assess the prevalence of LTE in the stools of schoolchildren, identify risk factors for infection and ascertain the trematode species, all with a focus on *Fasciola* spp.. We performed a cross-sectional study among the schoolchildren, examined cattle droppings in and around Damrei Chhlang village and inspected cattle livers in a local slaughterhouse.

2. Materials and methods

2.1. Ethical considerations

Ethical clearance for this study was obtained from the Ethics Commission of Basel (EKBB; reference no. 159/11) and from the National Ethics Committee for Health Research (NECHR), Ministry of Health (MOH) in Phnom Penh, Cambodia (reference no. 30/NECHR). Before field work started, the provincial and district health authorities, village and school authorities and parents were informed of the study and, in turn, working permission was granted. Prior to enrolling each participant, written informed consent was obtained from the parents, legal guardian or the participant him/herself if they were of legal age. In cases of illiteracy, the informed consent was signed by fingerprint before the village chief, who signed as a witness.

2.2. Study design, population and area

The study was carried out in Central Kandal province, which surrounds Phnom Penh. It is inhabited by 1.2 million people and divided into 11 districts. Between May and June

2011, a cross-sectional study was carried out among schoolchildren at the Damrei Chhlang village primary school, S'ang district (11°21'29.43''N, 104°59'18.05''E), a district of 1,831 people.

Another cross-sectional study aimed to estimate the prevalence of fascioliasis in cattle droppings in the villages surrounding the Damrei Chhlang primary school (S'ang district). These villages included Damrei Chhlang and Preaek Khmaer, (11°21'21.70''N, 104°58'59.33''E), both located in S'ang Phnum commune; and Preaek Run (11°21'16.30''N, 104°59'46.54''E), located in Preaek Koy commune. Additionally, an abattoir study of a small facility that slaughtering local cattle was conducted in Preaek Koy commune.

2.3. Procedures in the field

On day one, enrolled study participants assembled at the community hall next to the school. A trained interviewer questioned each child about potential risk factors for infection and about experiences of ill-health. Subsequently, a physician assessed participants' general health status and clinical symptoms by using a standardized assessment form. Additionally, a nurse drew a venous blood sample of 5 ml from each child. The blood samples were stored in a cooling box at about 5-10°C until they reached the laboratory of the National Center for Parasitology, Entomology and Malaria Control (CNM) in Phnom Penh.

A pre-labeled stool container was given to each child, along with instructions on how to fill it. Children were asked to collect morning stools to ensure freshness of the stool samples. The following morning, the filled container was collected and a new pre-labeled

container was provided. Three stool samples were collected from each child over four consecutive days. Each day after collection, containers were directly transported to the laboratory of the CNM for analysis.

The stool containers for cattle droppings were distributed among randomly selected cattle owners, each of whom collected one fresh stool sample from every one of their cattle. Containers were collected in the morning, the day after distribution, and directly transported to the laboratory of the CNM.

The butcher at the study abattoir examined the livers of slaughtered cattle on a continuous basis for meat quality assurance. For study purposes, he recorded in a diary the total number of slaughtered cattle and of *Fasciola* spp. infected livers.

2.4. Laboratory procedures

For each of the three stool samples per child, one Kato-Katz thick smear (Katz et al., 1972) was prepared directly after the samples arrived in the laboratory. After a half an hour clearance time, the smears were examined under a light microscope (magnification 400x) for intestinal helminth eggs. The number of eggs per parasite species was counted and recorded.

From each of the three stool samples per child, approximately 1 gram was preserved in 15 ml sodium acetyl formalin (SAF) fixative for analysis by formalin-ether concentration technique (FECT) (Marti and Escher, 1990). Four weeks after collection, the fixed samples were processed and examined under a light microscope (magnification 1000x) for intestinal helminths and protozoa.

From the first stool sample per child, approximately 0.5 gram of fresh stool was preserved in 70% ethanol for later molecular analysis using real-time quantitative Polymer chain reaction (PCR) to diagnose *Fasciola* sp. (Alasaad et al., 2011) and *Fasciolopsis buski* infections (unpublished, developed by Dr. med. Stefanie Kramme, Swiss TPH). The preserved samples were sent to the Swiss TPH laboratory in Basel, Switzerland, where molecular analysis was carried-out about two months later. First, from each preserved stool sample, 200 µg was washed twice with phosphate buffered saline (PBS) to eliminate the ethanol. Subsequently, DNA was extracted with the QIAamp DNA Stool Mini Kit (QIAGEN, USA). The extracted DNA was frozen at -20°C until PCR was performed, one week later. On 0.5 gram of the first stool samples from each child, a commercially available *F. hepatica* coproantigen enzyme-linked immunosorbent assay (ELISA test) (Bio-X, Belgium) was performed at the CNM laboratory.

Upon arrival, blood samples were stored at room temperature to clot. Subsequently, the blood samples were centrifuged and the serum was frozen and stored at -20°C at the CNM laboratory. The frozen samples were sent to the Diagnostic Center of the Swiss TPH, Switzerland, where an ELISA for *Fasciola hepatica*-antibodies was performed two months after sample collection. Sera samples with a positive result were retested with an immunofluorescence antibody test (IFAT) for confirmation. All sera samples with a positive or critical (close to positive) result were retested with a helminth screening ELISA (unpublished, Dr. Hanspeter Marti, Swiss TPH), which included *Fasciola hepatica*, *Strongyloides stercoralis*, *Schistosoma* spp., *Burgia malayi*, *Wuchereria bancrofti*, *Echinococcus* spp.*, Toxocara* spp. and *Trichinella* spp..

Cattle droppings were stored at 5°C in a fridge at the CNM laboratory until analysis. The next day, cattle droppings were examined by the same *F. hepatica* coproantigen ELISA test (Bio-X, Belgium) that had been used to examine the stool samples from children. Identical procedures were used, only the weight of stool was increased from 0.5 gram to 2 grams. Within 48 hours of collection, cattle droppings were examined a second time by cup sedimentation. Approximately 10 grams of fecal material was mixed with NaCl 0.9% and filtered through a double layer of gauze. The cup was filled with NaCl 0.9% and left to stand for one hour. Three slides of the sediment were examined under a light microscope (magnification 400x) for *Fasciola* spp. eggs. The left over sediment was centrifuged, mixed with SAF-solution and ether, vigorously shaken, centrifuged again and examined again under the light microscope (magnification 400x).

2.5. Risk factor assessment

Potential risk factors for LTE infection were assessed, using a two-part questionnaire. First, a questionnaire was administered to each schoolchild to collect individual information about basic demography, eating habits and personal hygiene. Second, a household questionnaire was administered to the head of household to obtain demographic information for the head of household and for the participant, as well as information about the participant's recent medical history, eating habits and food consumption, household assets and water and sanitation facilities. The questionnaire provided information about exposure to potential risk factors, general health information, and household. A physical examination of each participant was conducted by qualified CNM staff to collect information about his/her general physical condition, recent

medical history and other clinical observations. By understanding participants' general health and the household conditions in which they live, the questionnaire and physical exam provided information on exposure to potential risk factors for trematode infection.

2.6. Data management and analysis

All data were double entered into Epidata v3.1 (EpiData Association; Odense Denmark), and validated and analysed by Stata v10.1 (Stata Corporation; College Station, TX, USA). The analysis included all schoolchildren with complete individual and household questionnaires, a 5ml venous blood sample and at least one stool sample examined by Kato-Katz, FECT and PCR analysis.

The gold standard for diagnosing LTE- and co-infections is the compiled results of three Kato-Katz slides and three FECT analyses. Concomitant, a child was defined as LTE positive (outcome) if at least one LTE was observed in either the Kato-Katz and/or FECT examination. The same definition was applied to co-infections. Age groups and sex were tested for differences in LTE or co-infection prevalence with a chi square likelihood ratio test (LRT) or with Fisher's exact test. The diagnostic performance of Kato-Katz vs. FECT for LTE detection was compared by using the McNemar test or the exact McNemar test. Arithmetic mean of eggs per gram (epg) of feces was calculated for LTE and co-infections. Additionally, the real prevalence of LTE and co-infections was estimated by a prediction model (Marti and Koella, 1993).

Anthropometric measurements were calculated and stratified for age and sex. Gender distribution, anthropometric measurements and fever were tested for differences with a binomial test (0.5), one sample t-test and one-way ANOVA, respectively.

Each participant's socio-economic status was estimated using a household-based asset approach, in which the study population was stratified into wealth index tertiles: (i) most poor (ii) poor and (iii) least poor. Wealth index tertiles were calculated by principal component analysis (PCA), using the following household assets: cattle/horse cart, farm vehicle with engine, car, motorbike, bicycle, mobile phone, television, radio, electric fan, current generator, direct electricity, animal ownership (chickens, ducks, pigs, cows) and food security (rice field, vegetable garden). The unadjusted first principal component (PC) accounted for 12.5% of the total variability and its weights were used to calculate the household index score. Finally, asset scores were summarised and assigned to each participant (Houweling et al., 2003).

A univariable logistic regression analysis was performed to associate potential risk factors with the presence of LTE (outcome). Odds ratios (OR), 95% confidence intervals and p-values were calculated. Variables with a p-value of less than 0.25 in the univariable analysis were selected as possible predictors in the multivariable logistic regression analysis.

The full multivariable model with all preselected variables underwent backward selection and was rated by the use of the Bayesian information criterion (BIC). For the final multivariable model odds ratios (ORs), 95% confidence intervals and p-values are reported and factors with a p-value of less than 5% were considered as significant.

The gold standard diagnosis of fascioliasis in living cattle relied on the combined results from two stool examinations (cup sedimentation and copro-ELISA, Bio-X, Belgium). Cattle were defined as *Fasciola* spp. positive (outcome) if at least one *Fasciola* spp. egg was observed in one stool examination.

3. Results

3.1. Schoolchildren's characteristics

Of the 257 schoolchildren enrolled, 228 (88.7%) had complete data records (household questionnaire, individual questionnaire, blood sample and at least one stool sample) and were used in the final analysis (Figure 1). All participating schoolchildren lived in the Damrei Chhlang village.

The children ranged in age from 5 to 18 years, with a median of 11 years and the following age structure: 5-7 years (16.7%), 8-10 years (29.4%), 11-13 years (38.2%) and 14-18 years (15.8%); 50.9% were male. Overall mean height was 127.3 cm (range 95-160 cm, SD 14.7 cm) and males were not significantly taller compared to females (p=0.982). Mean weight of males and females did not differ significantly (p=0.985) and overall mean weight was 24.9 kg (range 10.5-50.0 kg, SD 7.8 kg). Mid Upper Arm Circumference (MUAC) was on average 17.2 cm (range 10.5-26.0 cm, SD 2.2 cm) and did not differ significantly between the sexes (p=0.770). Mean overall body temperature was 36.1°C (range of 34.0-37.8°C, SD 0.6°C) and did not differ between sexes (p=0.847) or age groups (p=0.580).

3.2. Infection with large trematode eggs and associated clinical signs

In almost half of the study participants (46.5%), LTE were observed in at least one stool examination. There was no significant difference between the sexes (p=0.608) but infection prevalence differed between age groups (p=0.037) and was highest among the the group aged 11-13 years (Table 1).

The prevalence of LTE as determined by the Kato-Katz technique (KK) was 21.9% and 36.4% as determined by FECT. There is a significant difference between these two tests (p<0.001) with a ratio (KK/FECT) of 0.602 (95% CI 0.460-0.789). Considering the combined results of three Kato-Katz slides and three FECTs (n=203) as the gold standard for detecting LTE, the cumulative sensitivities for the first, second and third Kato-Katz slide were 30.2%, 37.7% and 47.2%, respectively and for the first, second and third FECT 37.7%, 67.0% and 78.3%, respectively.

Blood samples were tested by serum ELISA and *Fasciola*-antibodies were detected in two samples. LTE were not observed in these two samples via KK or FECT. Subsequently, the two positive samples were retested by IFAT, which yielded negative test results. Considering the negative IFAT result, the two positive samples and those samples with a critical (close to positive) value after the first serum ELISA were retested with a helminth screening ELISA. No *Fasciola*-antibodies were detected with this test. Using copro-ELISA, *Fasciola*-antigens were identified in six participants. Out of these six participants, only two had observable LTE in the stool while one other had a positive serum ELISA result.

Of the 221 human stool samples tested with the *Fasciola*-specific PCR, one showed a positive result. The *F. buski*-specific PCR was negative for all stool samples. The participant with the positive PCR result had neither LTE in the stool (Kato-Katz, FECT) nor a positive result in serological ELISA.

Study participants were clinically examined and questioned about the presence of clinical signs and symptoms in the two weeks prior to examination, particularly those relating to the suspected parasitic infections. A number of symptoms were reported, including

abdominal pain (64.5%), diarrhea (54.0%), fever (52.6%), pain in the right upper quadrant (25.0%), vomiting (21.9%), itchy skin (15.8%) nausea (14.9%) and weight loss (11.8%). Clinical signs observed by study physicians included generalised rash (2.6%), pale skin color (2.2%), pale subconjunctiva (2.2%), jaundice (1.8%) and poor general condition (3.1%). Only one participant suffered from splenomegaly and none of the participants suffered from ascites or hepatomegaly. Statistical analysis (univariable and multivariable regression analysis) showed that none of the clinical signs and symptoms were associated with LTE infection.

3.3. Other intestinal parasitic infections

Overall, 70.2% of the participants were diagnosed with an intestinal parasitic infection (Table 1). A significant difference in prevalence was observed between age groups (p=0.026). The highest infection prevalence of more than 75% was observed in the groups aged 8-10 years and 11-13 years. There was no significant difference between sexes (p=0.644).

Besides LTE, another trematode, *O. viverrini* was diagnosed in the study population. The observed prevalence of *O. viverrini* in the study population did not differ between age groups (p=0.540) or by sex (p=0.355). Nematode infections presented the biggest diversity, with five different species observed. Most common were hookworms, for which no significant age (p=0.214) or by sex (p=0.382) difference was observed. *Ascaris lumbricoides* had the lowest prevalence, which did not differ significantly between age groups (p=0.866) or by sex (p=0.679). Additional nematodes diagnosed were *Trichuris trichiura, Enterobius vermicularis* and, in one participant, *S. stercoralis* larvae.

Hymenolepis nana, Taenia sp., *Entamoeba histolytica / dispar / moshkovskii, Gardia lamblia, Entamoeba. coli* and *Isospora belli* were also detected.

Helminth and intestinal protozoa multiparasitism was common among the participants. Of the participants, 36.4% were infected with a single parasite and 33.8% with more than one species. Participants were infected with two, three and four different parasites species, with a prevalence of 22.8%, 8.3% and 2.6%, respectively.

3.4. Risk factors for an LTE infection

In the univariable logistic regression, of the variables describing the demographics of the study participants, only age (11-13 years) was significantly associated with the observation of LTE (p=0.012) (Table 2). Of the variables describing the anthropometric measurements and fever, none were significantly associated with the observation of LTE. Body temperature, body weight and mid upper arm circumference met the inclusion criteria for the multivariable logistic regression analysis.

Variables pertaining to the participant's eating habits, consumption of raw aquatic plants (p=0.029) and consumption of fermented fish sauce (p=0.032), were significantly associated with the observation of LTE. The variables raw fermented fish sauce (p=0.210), pork meat (p=0.227) and aquatic plants (p=0.238) met the inclusion criteria for the multivariable logistic regression analysis. Of the variables describing the participant's hygiene practices, none were significantly associated with the outcome. Only daily bathing in river or pond (p=0.178) met the inclusion criteria for the multivariable logistic regression analysis.

In the multivariable logistic regression analysis, the consumption of raw aquatic plants (OR=3.3, 95% CI 1.3-8.5, p=0.013) and fermented fish sauce (OR=2.1, 95% CI 1.0-4.4, p=0.046) were significantly associated with the observation of LTE in the stool. The consumption of raw aquatic plants resulted in a three-fold risk increase for an LTE infection, confirming the finding of the univariable logistic regression analysis where the consumption of raw aquatic plants showed the same trend. The consumption of fermented fish sauce doubled the risk for an LTE infection, consistent with the univariable logistic regression analysis, which also showed a significant positive association with LTE infection. Of the remaining variables in the multivariable logistic regression model, i.e. daily bathing in river or pond, itchy skin, generalized rash, general condition, body temperature and mid upper arm circumference, none were significantly associated with the presence of LTE in stool.

3.5. Fascioliasis study in cattle

For the study, a sample size of 200 cattle was estimated and 222 cattle were enrolled. The analysis included 205 cattle (Preaek Run (58), Damrei Chhlang (59) and Preaek Khmaer (88)) with complete data records, i.e. 1 stool sample, 1 cup sedimentation and 1 ELISA test. Of the 205 cattle analysed, there were 84 males and 121 females. The overall prevalence of cattle fascioliasis was 88.8%, using the gold standard diagnostics. Among males, the prevalence was 88.1% and 89.3% among females.

During the 17-day observation period, a total of 191 cattle were slaughtered and their livers were examined for *Fasciola* spp. flukes, resulting in the detection of 35 (18.3%) cattle livers infected with *Fasciola* spp.

4. Discussion

In this study, we showed that fascioliasis is endemic in the Damrei Chhlang primary school environment. An 18.3% prevalence rate was observed among the livers of slaughtered cattle, while an average prevalence of 88.8% was diagnosed from bovine stool from surveyed villages. Our observation is consistent with another study conducted in Kandal province in 2001 and 2002, which found an infection prevalence of 85.2% (Tum et al., 2007). This high bovine fascioliasis prevalence was predicted by a GIS risk map developed by Tum and colleagues (Tum et al., 2004). Therefore, given the environmental contamination with *Fasciola* spp. eggs, the proximity of cattle to villagers and the raw food consumption of villagers, zoonotic transmission of the parasite from cattle to human is possible in this area.

In schoolchildren and adolescents, we found a high prevalence (46.5%) of LTE in examined stool samples. Two participants tested positive through an ELISA test on blood serum, while one participant's DNA tested positive via a PCR for *Fasciola* spp.. Our risk factor assessment showed that the consumption of raw or undercooked aquatic plants was a significant risk factor for LTE infection. Furthermore, the veterinary copro-ELISA detected *Fasciola*-antigens in stool samples from six (of 228) human participants, two of which had observable LTE in the stool.

The prevalence of LTE among study participants was very high and cannot be explained by the *Fasciola* spp. positive results of the ELISA test on serum or the PCR. In addition, the results of the ELISA test on serum were controversial. The first ELISA detected two *Fasciola* positive cases. Both cases had a negative confirmatory IFAT. It must be

mentioned that the antigen used for the ELISA test on serum was crude worm extract of *F. hepatica*. Therefore, the detection of *F. gigantica*-antibodies was only partially or not at all possible. Of the two participants with positive ELISA test results (on serum), one also tested positive by copro-ELISA, but no LTE were found in the stools of either participant. This finding is consistent with findings in Vietnam where eggs in the stool were observed in only 14 of 285 fascioliasis cases (Hien et al., 2001). Finally, only two participants had a *Fasciola* spp. positive PCR result. Given these results, we conclude that it is very unlikely that the observed LTE are *Fasciola* spp. Eggs, but rather belong to *Fasciolopsis* or *Echinostoma* trematode species.

Generally, the presence of *Echinostoma* spp. in Southeast Asia is well known (Chai et al., 2009). Different *Echinostoma* species have been reported in Cambodia's neighboring countries: Lao PDR (Chai et al., 2012; Sohn et al., 2013), Thailand (Radomyos et al., 1998; Tungtrongchitr et al., 2007; Waree et al., 2001) and Vietnam (Chai et al., 2011). At the beginning of the 2000s, the first *Echinostoma* spp. infections were reported in Cambodian schoolchildren, with prevalences ranging from 4.8% to 15.6% (Lee et al., 2002; Park et al., 2004).

In 2011, Sohn and colleagues demonstrated the presence of *Echinostoma revolutum* in Pursat province and *Echinostoma ilocanum* in Oddar Meanchey province, Cambodia, by noting the distinct morphological characteristics of adult flukes recovered from human patients (Sohn et al., 2011a, 2011b). Their published pictures of *E. ilocanum* eggs observed during Kato-Katz analysis were strikingly similar to the LTE observed during our Kato-Katz analyses (Figure 3), suggesting that the LTE observed in our study were

most likely due to *Echinostoma* spp. infection. We did not systematically collect LTE measurement data and hence, cannot compare it with Sohn et al.'s measurement data. Surprisingly, the risk factors identified in our study were not consistent with those for *Echinostoma* infection. In fact, we found that the consumption of raw aquatic plants and fermented fish sauce were significant risk factors for LTE eggs in stool. The consumption of raw aquatic plants is a well-known way of acquiring a *Fasciola* spp. or *F. buski* infection. However, humans acquire *Echinostoma* spp. infections in Cambodia by consuming uncooked snails, clams and freshwater fish (Sohn et al., 2011a, 2011b). In our study, the consumption of raw or undercooked fish was not reported by any participant. Cooked fish consumption was reported by nearly all study participants and it was not significantly associated with an increased risk of LTE in stool in the univariable analysis. It is evident that some of these participants were consuming raw or undercooked fish, as *Opisthorchis viverrini* infection was detected in a considerable number of participants. This in-depth investigation documents once more the high degree of intestinal helminthic infection, multiparasitism and diversity in a semi-rural setting in Cambodia. Almost three-quarters (70.2%) of the study participants harbored a helminth infection. Multiparasitism was observed in one-third (33.8%) of the participants. Even higher rates were observed in rural Laos (Sayasone et al., 2011, 2009). Besides LTE infection, we diagnosed seven additional helminth species. At this point, it must be noted that *Opisthorchis viverrini* infection diagnosed through Kato-Katz analysis were also confirmed in FECT analysis. However, minute intestinal flukes (MIF) such as *Haplorchis taichui* have eggs very similar to *O. viverrini.* Given their similar morphological appearance, it is possible that some reported *O. viverrini* eggs might belong to MIF.

The prevalence of *S. stercoralis* and *E. vermicularis* in this study were underestimated due to the fact that Kato-Katz and FECT are not the appropriate diagnostic methods for these infections. The gold standard for diagnosing *S. stercoralis* is a combination of the Baermann technique and Koga-Agar cultures (Baermann, 1917; Koga et al., 1991) and for *E. vermicularis,* the scotch-tape anal swab technique (Cho and Kang, 1975). Hence, diversity of the helminthic infection is underestimated in our study.

Our findings on the cumulative sensitivities of Kato-Katz and FECT are in line with the observations of other authors: FECT is, in general, more sensitive than Kato-Katz for detection of LTE and the sensitivity of both techniques increases with multiple stool sampling (Glinz et al., 2010; Knopp et al., 2009, 2008).

As the applied *Fasciola* spp. PCR is published and tested for different areas (Alasaad et al., 2011), most likely the egg intensity was too low to be detectable or eggs were not present in the stool samples, as was observed in Vietnam (Hien et al., 2001). The use of 200 µg stool for DNA extraction may also have been too small an amount to reach an acceptable level of sensitivity. For the *F. buski* PCR, it is possible that the published ITS-2 sequence on GenBank differs from the ITS-2 sequence of the study site, thus, the primers used were unable to detect *F. buski* eggs or the observed LTE were not caused by *F. buski*. Further, for both PCRs, there is the possibility of inhibition in the stool samples.

5. Conclusion

It is unlikely that fascioliasis caused the observed LTE in humans. The detected prevalence of 46 LTE does not match the findings from the serum ELISA and PCR analysis. The most plausible cause is *Echinostoma* spp. Infections, which are reportedly endemic to Cambodia. However, risk factors for the transmission of *Fasciola* spp. and *F. buski* transmission are present. Hence, transmission may occur and public health services must remain vigilant.

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Figure legends

Figure 1: Flow chart of cross-sectional study on helminthic infections in Cambodian schoolchildren

Figure 2: Cumulative prevalence of combined Kato-Katz and FECT results for the number of examined stool samples

Figure 3: Large trematode egg in Kato-Katz fecal smear of Damrei Chhlang (Magnification 400x)

Table 1: Prevalence of intestinal parasitic infections in schoolchildren diagnosed by Kato-Katz and FECT

Intestinal Parasites prevalence (%)	Age in years				Sex		Overall	Estimated prevalence (SD)	Infection Intensity * Mean EPG (SD)
	5-7 (N=38)	8-10 (N=67)	11-13 (N=87)	14-18 (N=36)	male (N=116)	female (N=112)	N=228		
Overall intestinal parasites prevalence	55.3	77.6	75.9	58.3	68.8	71.6	70.2	78.1 (7.3)	na
Trematodes									
Large trematode eggs	31.6	47.8	56.3	36.1	44.8	48.2	46.5	66.1 (12.5)	37.0 (87.2)
Opisthorchis viverrini	7.9	6.0	3.5	8.3	4.3	7.1	5.7	na	38.7 (70.3)
Nematodes									
Ascaris lumbricoides	0.0	3.0	2.3	2.8	1.7	2.7	2.2	na	45.3 (51.4)
Trichuris trichiura	13.2	19.4	13.8	5.6	17.2	10.7	14.0	15.6 (5.5)	130.3 (174.7)
Enterobius vermicularis	10.5	11.9	4.6	5.6	6.0	9.8	7.9	20.1 (20.2)	94.0 (194.1)
Hookworm	15.8	32.8	26.4	33.3	30.2	25.0	27.6	33.6 (8.5)	134.6 (216.4)
Strongyloides stercoralis	0.0	1.5	0.0	0.0	0.0	0.9	0.4	na	na
Cestodes									
Taenia sp.	0.0	0.0	1.2	0.0	0.9	0.0	0.4	na	64.0 (-)
Hymenolepis nana	7.9	3.0	3.5	5.6	5.2	3.6	4.4	5.8 (3.8)	610.7 (1115.0)
Protozoa									
Entamoeba coli	0.0	1.5	1.2	0.0	1.7	0.0	0.9	na	na
Entamoeba histolytica/dispar/moshkovskii	5.3	9.0	4.6	0.0	4.3	6.3	5.3	na	na
Giardia lamblia	0.0	1.5	3.5	0.0	1.7	1.8	1.8	na	na
Isospora belli	0.0	0.0	0.0	2.8	0.9	0.0	0.4	na	na

* Infection intensity assessed in Kato-Katz examination only, EPG eggs per gram, SD standard deviation, na not applicable

Table 2: Association of risk factors with infection with large trematode eggs (univariable regression analysis, n=228)

Risk factors	% of infected	% of non-infected	OR	95% CI	P-value
Demographics					
Socio-economic status (versus most poor)					
poor	32.1	34.4	1.1	0.6-2.0	0.870
least poor	36.8	30.3	1.4	0.7-2.6	0.330
Age (versus 5 to 7 years)					
8 to 10 years	30.2	28.7	2.0	0.9-4.6	0.109
11 to 13 years	46.2	31.2	2.8	1.2-6.2	0.012
14 to 18 years	12.3	18.9	1.2	0.5-3.2	0.681
Sex (female versus male)	50.9	47.5	1.1	0.7-1.9	0.608
Work in a rice field within the last year (yes versus no)	59.4	54.1	1.2	0.7-2.1	0.418
Help for water collection (yes versus no)	65.1	67.2	0.9	0.5-1.6	0.736
Eating habits					
Aquatic plants (yes versus no)	81.1	75.2	1.5	0.8-2.8	0.238
Raw aquatic plants (yes versus no)*	17.0	7.4	2.6	1.1-6.0	0.029
Pork meat (yes versus no)	85.9	91.0	0.6	0.3-1.4	0.227
Raw pork meat (yes versus no)	0.9	0.8	1.2	0.1-18.7	0.920
Prawns (yes versus no)	34.0	27.1	1.4	0.8-2.4	0.258
Raw prawns (yes versus no)	1.9	0.8	2.3	0.2-26.0	0.493
Chicken/Duck (yes versus no)	72.6	67.2	1.3	0.7-2.3	0.374
Fish dishes (yes versus no)	99.1	97.5	2.6	0.3-25.8	0.402
Fermented fish sauce (yes versus no)*	86.8	75.4	2.1	1.1-4.3	0.032
Raw fermented fish sauce (yes versus no)	6.6	11.5	0.5	0.2-1.4	0.210
Crab (yes versus no)	33.0	35.3	0.9	0.5-1.6	0.724
Raw crabs (yes versus no)	11.3	13.9	0.8	0.4-1.7	0.555
Cattle meat dishes (yes versus no)	38.7	38.5	1.0	0.6-1.7	0.981
Raw cattle meat dishes (yes versus no)	1.9	0.8	2.3	0.2-26.0	0.493

Personal hygiene habits

Usual places of defecation

Toilet (yes versus no)	75.5	76.2	1.0	0.5-1.8	0.894
Behind the house (yes versus no)	17.9	18.9	0.9	0.5-1.8	0.857
Forest (yes versus no)	5.7	5.7	1.0	0.3-3.0	0.980
Into the water (yes versus no)	0.9	0.8	1.2	0.1-18.7	0.920
Hand washing before eating the last time (yes versus no)	78.3	82.0	0.8	0.4-1.5	0.488
Hand washing before eating the last time (yes versus no)	64.2	62.3	1.1	0.6-1.9	0.772
Using soap for hand washing (yes versus no)	37.7	43.4	0.8	0.5-1.3	0.382
Daily bathing in river/pond (yes versus no)*	49.1	40.2	1.4	0.8-2.4	0.178

*included in the multivariable logistic regression